Cell density-dependent regulation of tumor necrosis factor α gene expression in a human hepatoma cell line

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Received June 27, 2007; Accepted August 17, 2007

Abstract. Human tumor necrosis factor α (TNFα) is a pro-inflammatory cytokine expressed in many cell types. Although the TNFα gene expression in human hepatocytes has been detected previously, its regulation is not well understood yet. In this study, we demonstrated that TNFα gene expression in human hepatoma cell line, huH2-2, was activated as a function of cell density. TNFα mRNA expression was low in the low-density culture, while significantly high expression was detected in the high-density culture. Moreover, stability of TNFα mRNA was not changed by cell density, eliminating a possibility of post-transcriptional regulation. Antibody neutralization against human TNFα had no significant effect on the TNFα mRNA expression. A cellular factor for the TNFα gene expression is suggested to be accumulated in the high-density cells. Data indicate that the level of TNFα gene transcription is elevated by a cellular factor in a cell density-dependent manner without influencing the TNFα secretion under the present cell-culture conditions used.

Introduction

TNFα is a multifunctional cytokine with a vast spectrum of physiologic and pathophysiologic functions (1-3). TNFα is mainly produced by hematopoietic cells, such as macrophages and infiltrating monocytes at the site of inflammation. Because of the dramatic increase of TNFα secretion after stimulation, hematopoietic cells have been employed to understand the details of TNFα gene regulation. However, accumulating evidence indicates that many other cells are also capable of expressing TNFα (4-7). Gonzalez-Amaro *et al* have reported the high TNFα expression by hepatocytes in the liver of patients chronically infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) (4). They demonstrated the involvement of TNFα produced from liver parenchymal cells to cellular destruction that leads to organ failure. Furthermore, a low level of TNFα expression in non-infected normal hepatocytes was observed. This showed the existence of a stimulus for hepatocytes' TNFα expression other than viral proteins.

TNFα can be induced in response to various agents including bacterial lipopolysaccharide, phorbol esters, exotoxins, lymphokines and virus infection (8-10). Stimulus-specific (8) and cell-type-specific (9) regulation of the TNFα gene have been reported by the assembly of transcription factors to the promoter region. Post-transcriptional regulation of TNFα expression has also been characterized and it is mainly due to the stability of the transcript modulated by the AU-rich element (ARE), that is located within the 3'-untranslated region of TNFα mRNA. ARE is capable of binding many regulatory proteins, causing the change of stability of the transcript (11-13). In addition, deletion of this element inhibits the degradation of the transcript (2). Thus, the human TNFα gene is regulated through complex pathways involving transcriptional and post-transcriptional mechanisms.

In this study, we examined the TNFα gene expression in the human hepatoma cell line, huH2-2, in which a single copy of the HBV genome was integrated and observed a significant modulation correlating with the cell density. Low TNFα mRNA expression was detected in the low-density culture, while significantly high expression was detected in the high-density culture. Furthermore, we demonstrated that this cell density-dependent TNFα gene expression correlated with a cellular factor probably accumulated in the high-density cells. Collectively, the present data indicate that cell density-dependent regulation of TNFα mRNA expression in the human hepatoma cell line huH2-2 provides important clues for understanding a regulatory mechanism of TNFα gene expression by increasing the level of TNFα mRNA in the high-density cells, without increasing the level of TNFα secretion.

Materials and methods

Cell culture. The human hepatoma cell line huH2-2 was derived from the cell line huH2, as previously described (14).

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Key words: tumor necrosis factor α gene, human hepatoma cell line, tumor necrosis factor α mRNA, cell density, transcriptional regulation, translational regulation, tumor necrosis factor α, hepatitis B virus, hepatitis B virus DNA integration

Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and glutamine in a humidified atmosphere of 5% CO_2. Cells were grown to sub-confluence and trypsinized, then replated to type I collagen-coated plates in fresh medium at various concentrations. After plating, the cells were allowed to recover for 20 h prior to starting the assays by adding fresh medium. The culture medium was changed every 24 h to maintain cell growth throughout the analysis up to 72 h. At each time point of the assays, the cells were washed with phosphate-buffered saline and harvested with a rubber policeman. The cell pellets were kept frozen at -80˚C until total RNA extraction. To determine the secreted TNFα quantity, cell culture supernatants were collected and centrifuged (1000 x g/5 min) to remove cell debris, and then subjected to the high sensitivity (h) TNFα ELISA system (Amersham Pharmacia Biotech.) according to the manufacturer's procedures. Actinomycin D (Act-D), Lipopolysaccaride (LPS) and recombinant human TNFα were products of Sigma. Monoclonal anti-human TNFα antibody was obtained from R&D systems.

RT-PCR. Total RNA was purified with an SV total RNA isolation kit (Promega) according to the manufacturer's instructions. Glyceraldehyde 3-phophate dehydrogenase (GAPDH) was employed as an internal control. The RNA concentration was measured using optical density at 260 nm. To detect full-length TNFα mRNA and TRAP-1 mRNA, RT-PCR was carried out with Takara RNA LA PCR kit (AMV) Ver.1.1, following the manufacturer's instructions. Briefly, 1 μg of the purified total RNA was reverse transcribed to cDNA independently in a 20-μl reaction (in final concentrations of 5 mM $MgCl_2$, 1 mM dNTP, 0.5 U RNase inhibitor, 0.25 U reverse transcriptase, 0.125 μM oligo dT-adaptor primer in RNA PCR buffer). Following reverse transcription, 80 μl of PCR reaction mixture (in final concentration: 2.5 mM $MgCl_2$, 2.5 U Takara LA Taq polymerase, 0.5 μM of each specific PCR primer in LA PCR buffer) were added directly to the same tube. Nucleotide sequences of the primers used in RT-PCR were as follows: TNFα forward primer, 5'-GCCAGCA GACGCTCCCTCAGCAAGGACAGCAGAG-3'; TNFα reverse primer, 5'-GCAGAGGCTCAGCAATGACTCACAGT TGG-3'; TRAP-1 forward primer, 5'-GCGTCACAAACTGGT GTCTGACGGCCAAGCACTG-3'; TRAP-1 reverse primer, 5'-GTCAACAAGTCCAGCAGCAATCATGGCGTTCTCG-3'; GAPDH forward primer, 5'-CATCGCTCAGACACCAT GGGGAAGGTGAAG-3'; and GAPDH reverse primer, 5'-CATGGCAACTGTGAGGAGGGGAGATTCAGT-3'. RT-PCR products were visualized by 1.0% agarose electrophoresis followed by 30 min of ethidium bromide staining.

Real-time RT-PCR analysis with the LightCycler. Real-time quantitative RT-PCR of TNFα mRNA or TRAP-1 mRNA was performed with a two-step PCR on the LightCycler instrument (Roche Diagnostics). To calibrate measurement deviation of each sample, GAPDH was employed as an internal standard. Of the purified total RNA, 1 μg was reverse transcribed independently by AMV reverse transcriptase (Takara) as described above.

For the LightCycler reaction, reverse transcribed cDNA (20 μl) was diluted with PCR grade water up to 200 μl. Diluted cDNA (10 μl) was added to 10 μl of reaction mixture (6 μl of water, 2 μl of primer, 2 μl of LightCycler Fast Start DNA Master SYBR-Green I). Capillaries were sealed, centrifuged and placed into the LightCycler rotor. LightCycler experimental protocol was carried out according to the manufacturer's procedure described in the primer sets. Mathematical procedures were performed using the LightCycler software 3.3 (Roche Diagnostics). Concentration of TNFα transcript was quantified. Ready-to-use amplification primer sets (Roche Diagnostics) were used in quantification of human TNFα and human GAPDH. Oligonucleotide primers used for real-time RT-PCR analysis were as follows: TRAP-1 forward primer, 5'-GATCAGATATACGAGAACG-3'; and TRAP-1 reverse primer, 5'-AAATAAAGCTCAAGGAGG-3'.

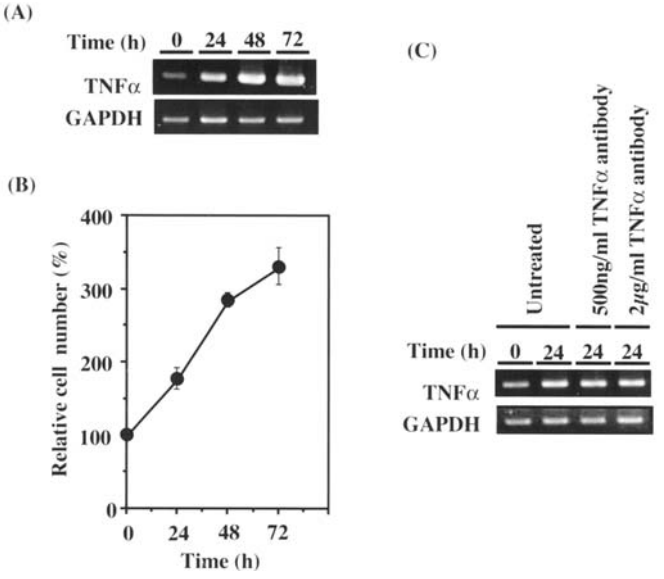

Figure 1. TNFα gene expression in human hepatoma cell line. (A) Visualized TNFα mRNA (1610 bp) of RT-PCR products at each time point. Cells ($2.0x10^6$) of human hepatoma cell line, huH2-2, were plated in 60-mm plates. Cells were harvested every 24 h and total RNA was extracted as described in Materials and methods. Of total RNA, 1 μg was subjected to RT-PCR for full-length TNFα mRNA detection and separated by 1.0% agarose gel electrophoresis. GAPDH mRNA (1145 bp) was used as an internal control. (B) Relative cell proliferation was calculated by counting live cells in trypan blue exclusion assay. Cell number at 0 time was indicated as 100%. Data represent mean values ± SD of three independent duplicate experiments. (C) TNFα mRNA was detected by RT-PCR after being cultured in the presence of monoclonal anti-human TNFα antibody (500 ng/ml or 2.0 μg/ml) for 24 h.

Results

TNFα mRNA expression is concomitantly modulated in human hepatoma cells during culture. To determine the level of TNFα gene expression in huH2-2 cells, total RNA was collected every 24 h for analysis during cell culture and TNFα mRNA was detected by RT-PCR. The level of TNFα mRNA in huH2-2 cells was low in the early stage, and then concomitantly elevated during 72-h culture (Fig. 1A). huH2-2 cells exhibited a constant growth rate throughout the 72-h culture (Fig. 1B), and cell death was not detected by trypan-blue staining (data not shown). The data indicate that TNFα gene expression is regulated in a cell density-dependent manner. Furthermore, treatment of huH2-2 cells with neutralizing antibody against

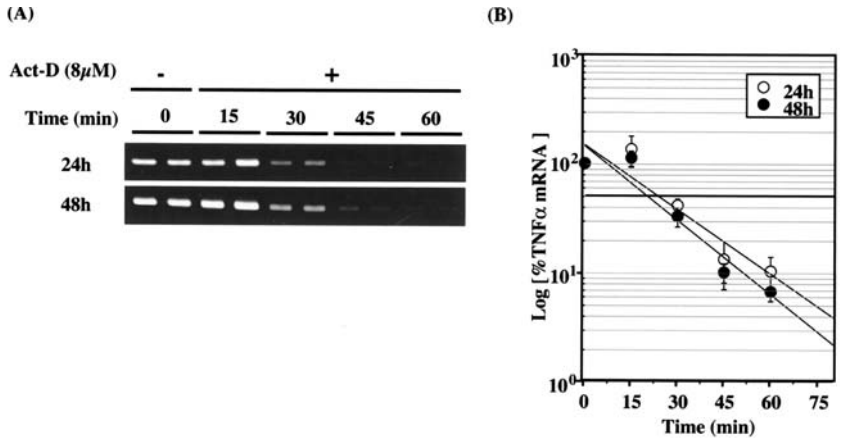

Figure 2. TNFα mRNA stability in two different cell culture times. The decay of TNFα mRNA was determined by quantifying TNFα transcript from Act-D (8 μM)-treated cells. (A) RT-PCR product of TNFα mRNA was visualized in 1.0% agarose electrophoresis. Total RNA was extracted every 15 min following Act-D treatment at 24 or 48 h. Duplicate samples are shown for each time point. (B) Mathematical quantification by two-step real-time PCR using the LightCycler instrument. Total RNA samples from A were used. TNFα mRNA levels at 0 time are indicated as 100%. Data represent mean values with standard deviation of a triplicate experiment.

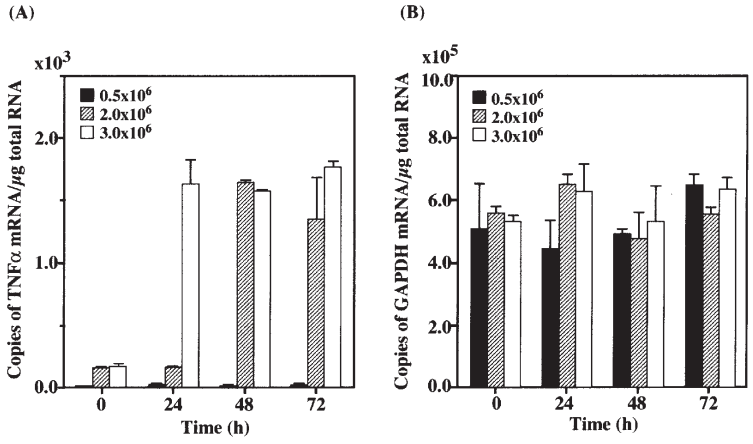

Figure 3. TNFα gene expression in different cell densities. (A) TNFα mRNA was quantified by real-time PCR as described in Materials and methods. Cells were plated into 60-mm plates at three different cell numbers, as indicated. Total RNAs were extracted every 24 h of culture time. (B) GAPDH mRNA was simultaneously measured as an internal control. Data represent mean values ± standard deviation of a triplicate experiment.

human TNFα had a minimal effect on the elevation of the TNFα mRNA level (Fig. 1C). Thus, a cellular factor other than TNFα in the huH2-2 cells is probably responsible for this elevation.

TNFα mRNA stability is not changed during culture. Since TNFα mRNA carries an AU-rich element within its 3'-untranslated region, which regulates the stability of the transcript (15), the decay of TNFα mRNA was measured by RT-PCR after addition of 8 μM Act-D, an RNA polymerase inhibitor, at two time points (24 or 48 h) (Fig. 2A). TNFα mRNA levels declined at a similar rate in both cases (Fig. 2B). Results indicate that the degradation rate of TNFα mRNA is independent of the cell density and that the level of intracellular TNFα mRNA is mainly regulated by the transcriptional mechanism in huH2-2 cells.

Level of TNFα mRNA expression depends on cell density. As a variety of cellular gene expression can be regulated by cell density, such as myelin (16), fibronectin (17) and insulin-like growth factor 1 (IGF-1) (18), we measured TNFα mRNA by two-step real-time PCR to determine whether TNFα gene expression is affected by the cell density. When huH2-2 cells were plated at low and high densities ($0.5x10^6$ and 2.0 or $3.0x10^6$ cells/plate, respectively), stimulation of TNFα gene expression was maintained in the high-density cells during 72-h culture (Fig. 3A), indicating that cell density regulates TNFα gene expression in huH2-2 cells. On the other hand, the level of GAPDH mRNA was not dependent on cell density (Fig. 3B). Thus, a cellular factor was probably accumulated up to a certain level in the high-density cells. As a matter of note, the culture medium was maintained to support cell growth at a constant level throughout the analysis up to 72 h.

The data suggest that the stimulation of TNFα mRNA expression is not a result of the cell abnormality caused by a prolonged culture.

TNFα secretion is not affected by the cell density. To elucidate the influence of cell density upon TNFα secretion in huH2-2 cells, we examined the secreted TNFα levels in low- and

Table I. TNFα secretion in the low- and high-density cultures of huH2-2 cells.

	TNFα (pg/ml)	
	Low density[a]	High density[b]
0 h	0.172±0.035	0.170±0.004
24 h	0.168±0.025	0.164±0.023
48 h	0.167±0.049	0.170±0.005

TNFα concentration in the culture medium of the huH2-2 cell was detected by the high sensitivity (h) TNFα ELISA system (see Materials and methods). [a]0.5x10^6 cells/plate, [b]3.0x10^6 cells/plate.

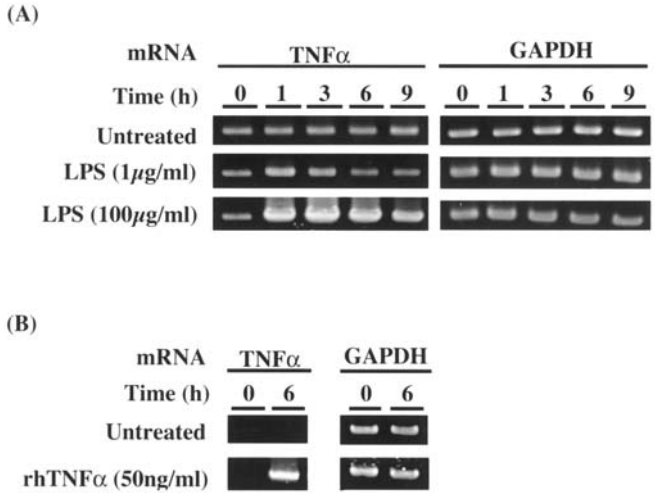

Figure 4. TNFα gene expression under stimulated conditions. TNFα mRNA was detected by RT-PCR at the indicated culture time after treating LPS (1 or 100 μg/ml) (A) or recombinant human TNFα (50 ng/ml) (B) in the culture medium and was visualized in 1.0% agarose electrophoresis.

high-density culture mediums of huH2-2 cells using a highly sensitive human TNFα ELISA system. Despite the increased level of transcript in the high-density cell culture, as shown in Fig. 3, there was no significant difference in the level of TNFα secretion between low- and high-density culture (Table I). Results indicate that the levels of TNFα mRNA and secreted TNFα are not related in huH2-2 cells.

Transient TNFα gene expression by LPS treatment. As transient expression of the TNFα gene is one of the characteristic features exhibited by extra-cellular stimulation in hematopoietic cells, we examined whether TNFα mRNA is transiently expressed in huH2-2 cells, when treated with LPS, a potent stimulator of TNFα gene expression. LPS was added to the cell culture at two different concentrations (1 μg and 100 μg/ml) and then TNFα mRNA was detected by RT-PCR. Dramatic increase of TNFα mRNA expression was detected as a peak after 1-h treatment of LPS in a dose-dependent manner (Fig. 4A). TNFα secretion was also detected following LPS challenge (unpublished data). The result indicated that TNFα

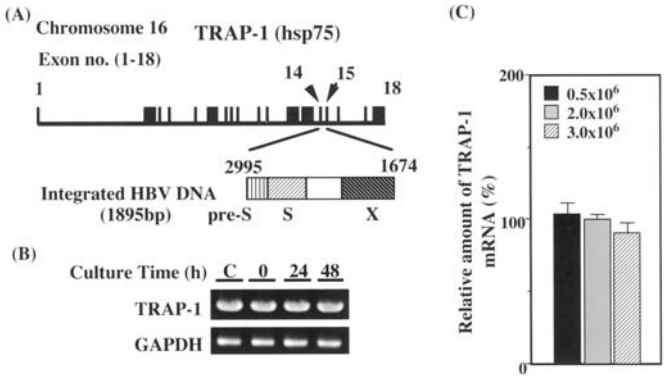

Figure 5. The effect of the HBV DNA integrated in the cellular DNA of huH2-2 cells. (A) Diagrammatic representation of the integrated HBV DNA within the TRAP-1 gene of the huH2-2 cell line. HBV DNA (1859 bp) is integrated within the intron region between exon 14 and 15. (B) Detection of human TRAP-1 mRNA (1671 bp). Total RNA extracted similarly to that shown in Fig. 1 was subjected to RT-PCR as indicated in Materials and methods. C indicates total RNA at 0 time of a hepatoma cell line (HepG2) with no HBV DNA integration used as a negative control. (C) The influence of cell density against TRAP-1 gene expression. Relative quantities of TRAP-1 mRNA normalized by the GAPDH mRNA are shown (values of 2.0x10^6 cells are indicated as 100%). Samples prepared as indicated in the legend of Fig. 3 were used for two-step real-time PCR. Data represent mean values ± SD of a triplicate experiment.

gene expression in huH2-2 cells is elevated temporarily and stimulated specifically in a dose-dependent manner, while the level of GAPDH mRNA was not affected (Fig. 4A). Data confirmed the intrinsic ability of huH2-2 cells to secrete TNFα. When huH2-2 cells were treated with human recombinant TNFα, potent activation of cellular TNFα mRNA expression was also detected (Fig. 4B), indicating that the signaling cascade from TNFα receptor to TNFα gene expression is operative in this cell line. Furthermore, the fold-increase of cell proliferation following 24-h incubation, in the absence and the presence of recombinant TNFα (50 ng/ml) was 1.54±0.075 and 1.51±0.111, respectively (data not shown). Thus, increased concentration of TNFα in the culture medium has a minimal effect on the cell proliferation of huH2-2 cells. These observations, together with the results in Figs. 1 and 3, show that huH2-2 cells are capable of exhibiting both transient and stable TNFα mRNA expression, which would be physiological responses to the environmental changes of the cell.

HBV DNA integration has no influence on cell density-dependent regulation of TNFα gene expression in huH2-2 cells. One interesting feature of the huH2-2 cell line is integration of single copy HBV DNA into the cellular DNA (14), where 1895 base-pair (bp) sub-genomic HBV DNA was found to be inserted within the intron region between exon 14 and 15 of the tumor necrosis factor receptor associated protein 1 gene [TRAP-1/hsp75 (19,20)] (Fig. 5A). Previously, the integration of HBV DNA was found to cause genetic changes in the host chromosome(s) of huH2-2 cells, and viral antigens were not detected (14). However, identical lengths of TRAP-1 mRNA were detected, when its structural feature was compared between huH2-2 cells and HepG2 cells, another human hepatoma cell line without HBV integration (data not shown). We quantified the levels of TRAP-1 mRNA expression in different

culture times (Fig. 5B) and cell densities (Fig. 5C) by real-time PCR. No significant difference was detected in their expression.

Discussion

Studies of TNFα gene expression in the past have provided a vast amount of evidence, and an ideal model was reported of how complex patterns of gene expression are established (8-10). In this study, we show that TNFα mRNA expression is regulated by cell density in human hepatoma cell line, huH2-2, while TRAP-1 or GAPDH gene expression was not affected, revealing a gene-specific regulation. This behavior was due to the transcriptional mechanism, since the stability of the transcript was not modulated by cell density manipulation. Previous studies have established that cell density can modulate cellular gene expression and thereby influence various functions of the cell (16-18).

It is known that HBV is a causative agent for acute and chronic hepatitis in humans, and its chronic infection is related to the development of hepatocellular carcinoma (HCC). The integration of HBV DNA occurs frequently in HCC tissues (14,21). However, integration is not essential for the life cycle of HBV. Here we observed apparently no influence of HBV DNA integration upon the cell density-dependent TNFα gene expression in established huH2-2 cells.

Cell density during liver development increases along with increase of homophilic interaction of hepatocytes at late gestation. Similar changes in liver marker gene expression have been observed through attempts to obtain an artificial liver system using various human hepatoma cell lines or primary cell cultures (22). For instance, increased albumin expression can be detected when primary mouse hepatocytes are cultured at high density (23). Therefore, cell density is an important feature to consider in *in vitro* experiments using liver cells.

We detected a lack of correlation between expression of TNFα mRNA and TNFα protein under varied cell densities. Other groups have also reported that such a lack of correlation is mainly due to the post-transcriptional mechanisms (15). Wolfert *et al* reported that treating cells with muramyldipeptide (MDP), the minimal structural subunit of peptidoglycan (PGN), induced TNFα mRNA expression, while TNFα translation was kept silent (24). However, this translational blockage is circumvented by the presence of either LPS or PGN. This indicates that MDP activates TNFα transcription, but not translation. Furthermore, IL-10 signal is known to inhibit TNFα mRNA translation via p38 MAPK activation (25). When phorbol 12-myristate 13-acetate (PMA) was administered to huH2-2 cells, TNFα mRNA expression and protein secretion were transiently activated within a few hours (unpublished data). This indicates that despite the huH2-2 cells being able to produce TNFα the post-transcriptional regulation is not activated, while TNFα gene transcription is activated in a cell density-dependent manner. However, an exact mechanism responsible for inhibiting post-transcriptional regulation of TNFα mRNA, which was expressed in response to the varied cell density, remains to be elucidated.

It is well accepted that the TNFα gene presents a transient behavior. Therefore, investigations at a rather short time range tend to be chosen in order to determine the influence of particular stimuli to TNFα gene expression (2). Potent induction of TNFα mRNA expression in huH2-2 cells was monitored within a short time following PMA challenge, which produced similar results to previous investigations. Thus, the transient behavior of this gene was not abrogated in this cell line. Moreover, even after the transient increase of TNFα mRNA by stimulation, elevated TNFα mRNA expression was detected at 24 h of culture time (Fig. 3). Taken together, our results indicate the presence of both mechanisms of transient and stable TNFα mRNA expression in huH2-2 cells, and the two cascades may not influence each other. In order to increase the TNFα mRNA expression, a higher dosage of LPS was necessary for huH2-2 cells, compared to previous studies with monocytes and macrophages (10). This is presumably caused by the difference between cell types of the expression levels of LPS receptors, such as CD14 or toll-like receptors.

Various environmental signals such as growth factors, extra-cellular matrix (ECM), cell-surface molecules on adjacent cells, or hypoxia can activate signaling pathways, which have an effect on the cellular gene expression (18,26). Table I shows low TNFα protein levels in the culture medium of huH2-2 cells. In addition, TNFα secretion was not influenced by the varied cell densities, and furthermore, neutralizing TNFα antibody treatment into the cell culture supernatant had a minimal effect on TNFα gene expression. This eliminates the possibility of feedback regulation by the secreted TNFα. A cellular factor other than TNFα probably accumulated up to a certain level in the high-density cells and must be responsible for the cell density-dependent TNFα gene expression (Fig. 3 and Table I). Preliminary characterization of a cellular factor revealed a low-molecular weight substance but further extensive studies are needed in future.

Each cell line has characteristics that reflect the tissue and cell type features it derived from. Differential expression of cellular genes in cancerous and normal cells is proposed to be involved in carcinogenesis, progression, or malignancy. Therefore, investigating a mechanism to increase the TNFα mRNA expression without influencing the level of TNFα synthesis or secretion may lead to further understanding of the distinctive features of human hepatoma cells.

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